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Most of the likely agents of bio-terrorism have profound effects on the host and, in particular, on the immune and inflammatory responses. We have proposed a broad-based approach to identify the unique "signatures" of infectious agents using host DNA microarrays. Because of the known diverse patterns of host cell interactions with these organisms, examination of the host transcriptional response has enormous potential to allow rapid diagnosis of infectious diseases in general and agents of bio-terrorism in particular. Key signature genes will serve as the basis for rapid diagnostic approaches that could be accessed when an outbreak is suspected. The agents under initial investigation are *Bacillus anthracis, Burkholderia mallei, Francisella tularensis*, multi-drug resistant *Mycobacterium tuberculosis* and *Yersinia pestis*. We have developed appropriate human and mouse models to explore the interactions of host cells with the pathogen. We have begun to characterize the pattern of genes activated or repressed by infection with virulent strains vs. attenuated organisms/mutants as an approach to ensure specificity. Using these data, we hope to identify disease markers to other bio-terrorism agents as well as other common infectious diseases to confirm the specificity of the diagnostic approach.

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Table of Contents

Introduction: Most of the likely agents of bio-terrorism have profound effects on the host and, in particular, on the immune and inflammatory responses. For many of these agents, pathogenesis has been studied at the cellular and molecular levels. These studies indicate that each specific organism has distinctive effects on the host immune and inflammatory cells that contribute to the unique clinical characteristics of the disease. These studies largely have focused on how the agent and its toxins and other constituents modulate host cell expression of individual cytokines and other molecules of interest as well as activation pathways. We have proposed a broad-based approach to identify the unique "signatures" of infectious agents using host DNA micro-arrays. Because of the known diverse patterns of host cell interactions with these organisms, examination of the host transcriptional response has enormous potential to allow rapid diagnosis of infectious diseases in general and agents of bio-terrorism in particular. The high through-put and scale of analysis possible with the use of DNA micro-array technology offers an ideal platform for these studies allowing us to identify and focus on a group of host genes that are most informative in this regard. Key signature genes will serve as the basis for rapid diagnostic approaches that could be accessed when an outbreak is suspected. Thus, recognition of specific genes that are expressed or repressed during these diseases will provide signature markers that can be used in related and alternative approaches for rapid diagnosis. This annual report summarizes our results to date with DNA microarray analysis of gene expression in a human whole blood infection model (*B. anthracis*) and a mouse infection model (*F. tularensis*), as well as preliminary examination of secreted proteins of *B. mallei*.

Body. There are five main aims that are relevant to all agents under initial investigation, *Bacillus anthracis, Burkholderia mallei, Francisella tularensis,* multi-drug resistant *Mycobacterium tuberculosis* and *Yersinia pestis*:

1. Develop human and mouse DNA chips to study transcriptional activation and repression by pathogens.

2. Develop appropriate *in vitro* models to explore the interactions of host cells with the pathogen and its toxins/constituents.

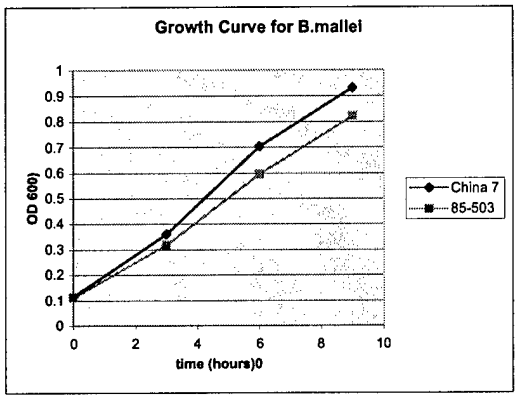
3. Characterize the pattern of genes activated or repressed:
    a. by infection with virulent strains vs. attenuated organisms/mutants as an approach to ensure specificity
    b. in infected cells from vaccinated vs. unvaccinated healthy individuals

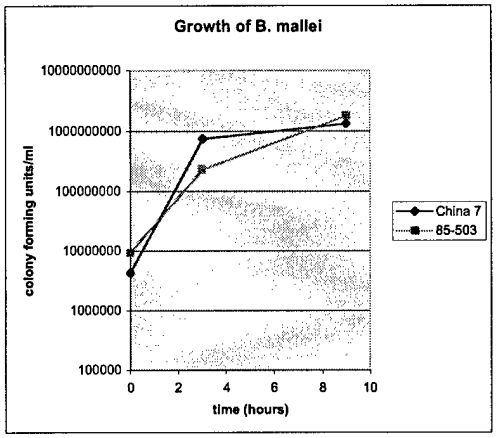
4. Using the mouse as an *in vivo* model of human infection, characterize changes in gene expression following infection with virulent and avirulent organisms.

5. Based on the above findings, develop DNA chips and assays for associated disease markers that focus on genes and their products that provide the best discrimination among these agents. Apply these DNA chips and assays for disease markers to other bio-terrorism agents as well as other common infectious diseases to confirm the specificity of the diagnostic approach.

Section 1. Whole blood infection: bacterial select agents (all protocols can be found in the appendix).

A. Growth curves of select agents. Each organism was grown in culture and viability was measured by plating dilutions of the culture throughout the growth cycle to determine colony-forming units. Representative curves, with *B. mallei*, are shown below (OD_{600} on the left; colony-forming units on the right). Each bacterial culture was grown to mid-log and used at a multiplicity of infection of 1 per macropahge/monocyte.





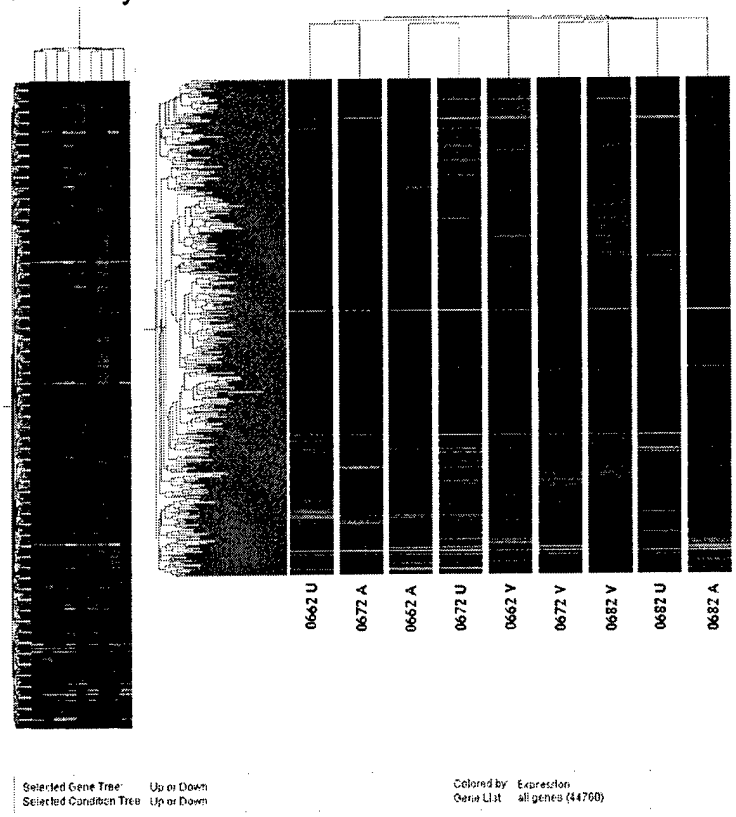
B. Whole blood Infections; RNA preparation. By September 1, 2003, bacterial infections with *B. anthracis, B. mallei, Y. pestis* and *M. tuberculosis* were completed. The bacterial infections were performed in quadruplicate, using blood from four different healthy volunteers. Using one virulent and one avirulent strain and four blood samples, we performed a total of 32 infections. Each infection was set up as follows: 240 mls of blood was drawn from a healthy volunteer. This blood was diluted 1:2 with RPMI and then aliquoted into 50 ml Nalgene Oakridge tubes with O-rings, at 20 mls per tube. This amount of blood provided sample for two bacterial infections at two timepoints, 3 and 8 hr. Each timepoint had the following conditions: uninfected, infected with an avirulent

strain, and infected with a virulent strain. Over the past year we have encountered challenges with RNA isolation from whole blood. After several protocols were tested, we optimized a protocol based on a method derived from Ambion, Inc. that successfully isolates clean and pure RNA from all whole blood samples (see attached protocols). At the specified timepoints, the blood was transferred to a 50 ml conical, centrifuged at 5000 rpm for 10 min, and the WBC was removed to a tube containing RNAlater (2:1). RNAlater protects RNA for different intervals depending upon storage. The samples were stored at -20 until they could be processed for RNA isolation. To date we have a total of 489 samples, of which 56 are still waiting RNA isolation and will be completed by November 1, 2003. 115 have been determined to be of good quality and transferred to the microarray facility for analysis. Once RNA is isolated, it is aliquoted for gel electrophoresis, spectrophotometry, and rtPCR validation. As of Oct 1, 2003, the microarray facility has received RNA samples from infections with *B. anthracis, B. mallei* and *M. tuberculosis*. The analysis of these RNA samples by Affymetrix is expected to be completed within 3 months (see below).

C. Whole blood RNA microarray analysis.

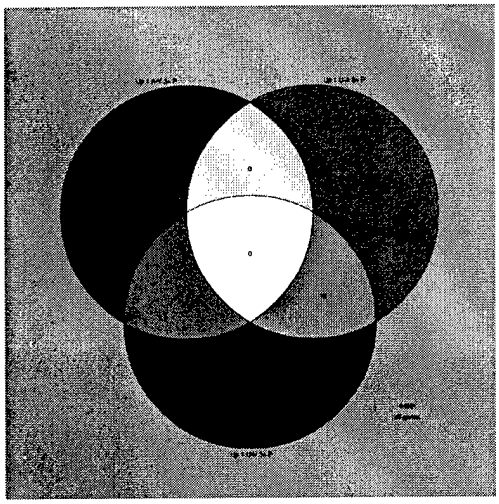
In our previous annual report, we reported preliminary data using oligonucleotide-based custom-printed DNA chips. Our subsequent microarray analyses using these chips showed such variability from individual to individual that we were changed our approach to the Affymetrix system. Using 3 hour time points from 3 infections with two strains of *B. anthracis* (V1B and Sterne strains), nine samples were examined, representing 3 patients with 3 infections (virulent, avirulent and mock-infected). The log ratio was calculated for each patient comparison (Uninfected v Avirulent, Uninfected v Virulent, Avirulent v Virulent). An up-regulated and a down-regulated list was made for each pair of conditions containing each gene whose log ratio was >= 1 (corresponding to a 2 fold change up) or <= -1 (2 fold change down) in 2 or more patients. These lists were then filtered so that only genes marked present in 3 or more of the 6 chips were involved. These genes were used for clustering and Venn Diagrams (below).

Cluster by Patient/Condition:

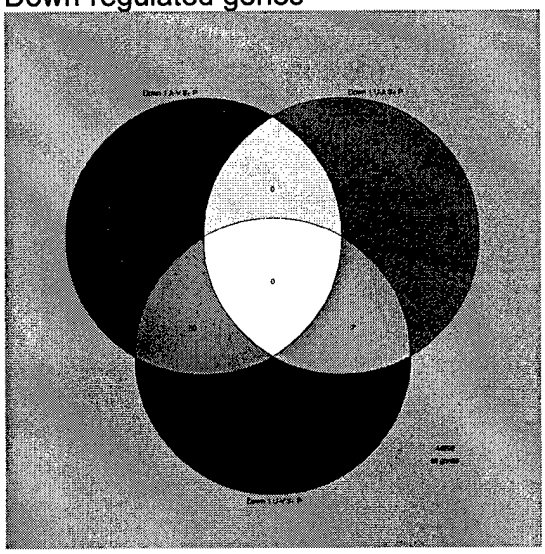


Venn diagrams:

Up-regulated genes:



Down-regulated genes



Interpretation of whole blood infection microarray data: 3 hour post-infection consensus data.

We have looked specifically at those genes arising from comparisons between uninfected cells and infected cells, that is, "UvA" (uninfected vs. avirulent) and "UvV" (uninfected vs. virulent). This approach represents the cleanest comparison, since is suggests what genes are actually changing expression in each infection. Furthermore, we have considered the data from the perspective of what the organism needs to do to survive and therefore, why the virulent strain is in fact, virulent. In particular, we have considered the effect of the organisms on those genes which constitute mechanisms of natural, host defense, the immune system.

In this context then, it is of interest to consider what effects are solicited by the avirulent strain, since it is effectively resisted by the host, and to ask which of those are absent in the virulent strain. The virulent strain may or may not actively repress these genes, but it has evolved some mechanism to prevent their induction and therefore, avoid the unwelcome attention of the human immune system, thereby being free to flourish in the human host.

Turning to the data from these experiments, we observe that early in the response to anthrax infection with the avirulent strain, whole blood cultures up-regulate a series of genes which indicate the initiation of an immune response. In particular, interleukin-6 and tumour necrosis factor are released in order to activate surrounding leukocytes and a broad range of C-C and C-X-C chemokines are activated, to attract other immune cells to the infected site. The activation of these genes is accompanied by upregulation of genes necessary for signaling into the cell, G-proteins and kinases in particular, and those associated with cellular activation in general. In other words, infection of human whole blood with avirulent anthrax stimulates the initiation of an immune response.

This contrasts markedly with the consequences of infection using the virulent strain. Here, none of the genes associated with immune activation are expressed. Nor are the complimentary signaling genes activated. Rather, the culture accepts the organism with no evidence of recognition and in consequence, the organism thrives. A consideration of the cellular genes downregulated following infection with either the virulent or avirulent strains re-enforces this point. In neither case is a key immune or inflammatory gene actively downregulated. More importantly, none is downregulated as a consequence of virulent infection over avirulent. Therefore, it can be tentatively concluded that those immunological genes upregulated in avirulent infection which are absent in virulent infection, are absent because they are actively targeted for repression by the virulent strain. These kinds of interference mechanism have been described in a number of organisms, for example, the smallpox virus. This successful pathogen expresses a number of proteins that

interfere with such immune mechanisms as complement activation, interferon signaling, and antigen presentation, among others. Recent work from with *B. anthracis* suggests that this bacterium's lethal toxin blocks key signaling pathways required for appropriate response in the host.

In future work, we will target these genes specifically (IL-6, TNF, the chemokines and their associated signaling genes) for confirmatory studies, using real-time, quantitative rt-pcr, and for future genetic studies. Furthermore, we shall combine these data (*B. anthracis*, 3 hr post-infection) with that forthcoming from 8 hr infections and data from *Y. pestis, B. mallei* and *F. tularensis* infections to produce customized microarrays. We shall develop these microarrays as tools for early, discriminatory detection of infection.

Section 2. *F. tularensis*/mouse infections
Sub PI: Raveche (collaborators: K. Elkins, FDA; T. Sellatti, Albany Med)
Results as of 9/29/03
Number of Microarrays: 26 Completed
5 In vitro Transcription (Last Step Remaining before Chip)

A. Experimental protocols:
Experimental Groups: (All mice identifications are indicated in **Table 1**)

I Groups A, B and C Infected either intraperitoneal or intradermal with 1 X 10^4 LVS strain of *F. tularensis*. Controls were injected with PBS. Strain; C57/Bl6. Mice killed at 4 days and RNA obtained from spleen. (See Table 1 for individual mice identification and date of microarray). Spleens stored in RNAlater and RNA extracted with Qiagen RNeasy. DNase employed, 15ug of RNA obtained as starting sample. In the case of samples A5 and C6 pooled spleens from the treatment group were employed. In experimental Group I there are two different experimental dates. Data were first analyzed separately and then pooled by treatment groups.

II. Groups H and K infected intranasal with 1 X 10^4 LVS and killed at 24 hours. Lungs obtained and stored in RNAlater. RNA extracted with Qiagen RNeasy with the modification that proteinase K step was added. 15ug of RNA obtained for all samples and no pooling was required.

III Group L experimental non-specific inflammation control with LPS. Mice were injected ip with LPS and sacrificed at 24 hours. Spleens were obtained, stored in RNAlater and then RNA extracted as for Experimental Group I.

Microarray Protocol:

Chips: Affymetrix murine chips were employed. For microarray of 10/02 the U74A chips were employed. For microarrays of 8/03 and 9/03 the newer 430A chips were employed. Preparation of labeled cRNA was as outlined in Affymetrix protocols. Test chips were used as screen and only cRNA which met the test chips requirement of coverage of at least 50% of test chip genes

Microarray Analysis:

Filters:

Relative Expression Filter: The list of genes analyzed was filtered. The total number of genes on the chip for U74A is 12,622. The total number of genes on 430 chips is 22,283. The first filter was the gene detection filter which required presence (above background) of gene relative expression as determined by Affymetrix analysis software. In the case of multiple samples from the same treatment group, the filtering required that the gene be present in the multiple samples. A gene could be present for instance in the control and absent in the treated group and this gene would still be represented in the first filter.

Fold Change Filter: The fold change represents the log base 2 change. Thus a fold change of 1 is equal to a 2 fold change a fold change of 2 is a 4 fold change. The second filter was the requirement for a 2-fold change between treated an untreated. Each group average values for fold change were employed for filtering. ***Report*** : List of genes, which pass the above two filters for each treatment group. Excel Files with the top 20 genes up and down regulated in tularensis infected group versus each control is presented in appendix CD. Total number of genes which meet the relative expression filter and the fold change for the U74A chips is 1032 genes and for the 430 Chip is 1486 genes (representing less than 10% of the total genes on the chip).

Gene Spring Analysis

Venn Diagram of Overlap: Experimental Group I employed two different infection routes. The overlap between these groups was identified for genes increased and decreased relative to control uninfected mice.. ***Report*** : (see **Table 3**)

Gene Function Analysis: The cluster analysis of condition trees identifies genes associated with *apoptosis, cytokines, cell signaling and immunity* were identified in the list of genes whose expression is altered by infection. ***Report*** : (see enclosed 8 clusters)

B. Overall Analysis.

In general, the reproducibility is good between samples of the same group. The lung has little gene expression related to immunity. Several candidate genes for

further investigation are indicated. There were two genes with surprising consistency in all infected animals regardless of route of infection or strain of animal employed. The two genes are listed below and are *clusterin* (increased in all RNA from all tissues studied from *F. tularensis* infected mice) and G7e protein.

Genes Altered in both lung and spleen of F. tularensis infected mice

	H - Average Infected Lung	K - Average Infected Lung	C vs S - Average Infected Spleen	C vs L - Average Infected Spleen	Description
1417821_at	1	2.03	-1.2	1.32	gb:BC027314.1Mm.22506 /s musculus, G7e protein
1418626_a_at	2.07	1.42	1.07	1.4	gb:NM_013492.1 Mm.196344Mus musculus clusteri

Raveche Table 1. Microarray Analysis in Mice Following *F. tularensis* Infection (LVS Strain)

A. Lethal tulie IP	DATE Microarray	Strain Organ	Chip
A5 (pool)	10/02	C57 Spleen	U74
A2	8/03	C57 Spleen	430
A4	8/03	C57 Spleen	430
B Sublethal tulie ID			
B1	10/02	C57 Spleen	U74A
B4	10/02	C57 Spleen	U74A
B7	8/03	C57 Spleen	430A
B10	8/03	C57 Spleen	430A
C. Control for ip/id			
C6 (pool)	10/02	C57 Spleen	U74A
C11	8/03	C57 Spleen	430A
C12	8/03	C57 Spleen	430A
C14	8/03	C57 Spleen	430A
H. Intranasal tulie C3H/			
H1	9/03	C3H/HeN lung	430A
H2	9/03	C3H/HeN lung	430A
H3	9/03	C3H/HeN lung	430A
H4	9/03	C3H/HeN lung	430A
HC Controls			
HC1	9/03	C3H/HeN lung	430A
HC2	9/03	C3H/HeN lung	430A
K. Intranasal tulie KO			

K1	9/03	C3H CD14KO lung	430A
K2	9/03	C3H CD14KO lung	430A
K3	9/03	C3H CD14KO lung	430A
K4	9/03	C3H CD14KO lung	430A
KC. Control in KO			
KC1	9/03	C3H CD14KO lung	430A
KC2	9/03	C3H CD14KO lung	430
L. LPS			
L14	10/02	NZB/BlN Spleen	U74A
LC. Control LPS			
L9	10/02	NZB/BlN Spleen	U74A
L13	10/02	NZB/BlN Spleen	U74A

Table 2.

File ID	Description	# Genes in Set	
Tulie Common.xls	Common to all infected mice/tissue	2	
Tulie Common SLR.xls	Common to all infected mice/tissue	2	
Control vs Sublethal 08-03.xls		654	
Control vs Sublethal 10-02.xls		226	
Control vs Lethal 08-03.xls		1092	
Control vs Lethal 10-02.xls		962	
Signal Transducer and Combo.xls		111	
Signal Transducer and Combo SLR.xls		111	
Signal Transducer and Combo 10- 2 SLR.xls		49	
Apoptosis Regulator and Combo.xls		30	
Apoptosis Regulator and Combo SLR.xls		30	
Apoptosis Regulator and Combo 10- 2 SLR.xls		20	
Cytokine and Combo.xls		54	
Cytokine and Combo SLR.xls		54	
Cytokine and Combo 10- 2 SLR.xls		24	
Immunity Protein and Combo.xls		75	
Immunity Protein and Combo SLR.xls		75	
Immunity Protein and Combo 10- 2 SLR.xls		52	
Control vs Lethal 10-02 Top 40.xls	Spleen Tularensis infection activated Gene Changes	20 increased 20 dereased	
Control vs Lethal 08-03 Top 40.xls	Spleen Tularensis infection activated Gene Changes	20 increased 20 dereased	
Control vs Sublethal 10-02 Top 40.xls	Spleen Tularensis infection activated Gene Changes	20 increased 20 dereased	
Control vs Sublethal 08-03 Top 40.xls	Spleen Tularensis infection activated Gene Changes	20 increased 20 dereased	
HS vs H 09-03 Top 40.xls	Lung Tularensis infection activated Gene Changes	20 increased 20 dereased	
KS vs K 09-03 Top 40.xls	Lung Tularensis infection activated Gene Changes	20 increased 20 dereased	

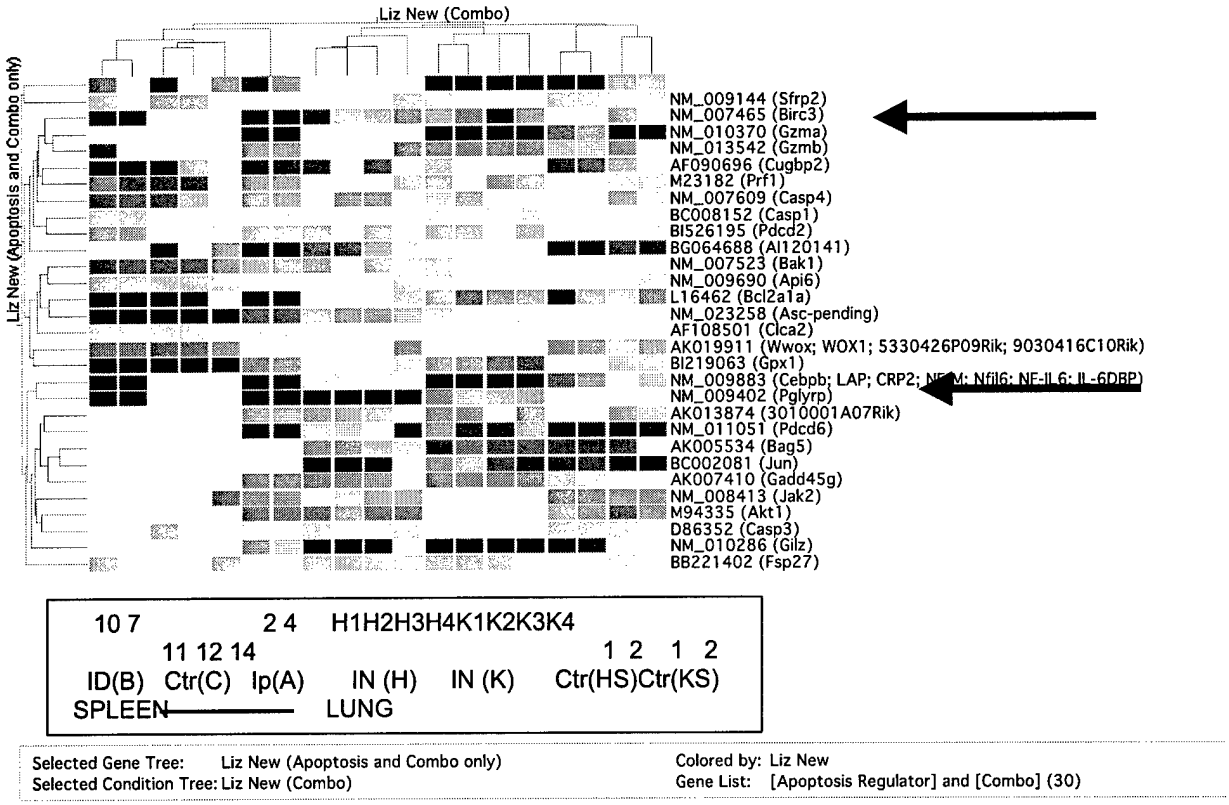
Table 3. Venn Diagram Generated List of Overlap Genes Data printed (Clone ID, common Name, unigene , abbreviated description)

Increased Spleen 10/02	Unique Lethal	(C6 v A5)	246
	Common Lethal and Sublethal	(C6 v A5)+ (C6 v B1/B4)	289
	Unique Sublethal	(C6 v B1/B4)	30
Decreased Spleen 10/02	Unique Lethal	(C6 v A5)	131
	Common Lethal and Sublethal	(C6 v A5)+ (C6 v B1/B4)	297

	Unique Sublethal	(C6 v B1/B4)	39
Increased Spleen 8/03	Unique Lethal	(C11//12/14 v A2/4)	246
	Common Lethal and Sublethal	(C11//12/14 v A2/4)+ (C11//12/14 v B7/B10)	289
	Unique Sublethal	(C11//12/14 v B7/B10)	30
Decreased Spleen 8/03	Unique Lethal	(C11//12/14 v A2/4	131
	Common Lethal and Sublethal	(C11//12/14 v A2/4)+ (C11//12/14 v B7/B10)	297
	Unique Sublethal	(C11//12/14 v B7/B10)	39
Increased Lung	Unique C3H/HeN	(HC1/2 v H1/2/3/4)	ND
	Common C3H and KO	(HC1/2 v H1/2/3/4) + (KC1/2 v K1/2/3/4)	ND
	Unique CD14 KO	(KC1/2 v K1/2/3/4)	ND
Decreased Lung	Unique C3H/HeN	(HC1/2 v H1/2/3/4)	ND
	Common C3H and KO	(HC1/2 v H1/2/3/4) + (KC1/2 v K1/2/3/4)	ND
	Unique CD14 KO	(KC1/2 v K1/2/3/4)	ND

Gene Spring Condition Trees: Dark indicates high expression and pale grey indicates low relative expression. The pattern of expression is similar between members of the same group.

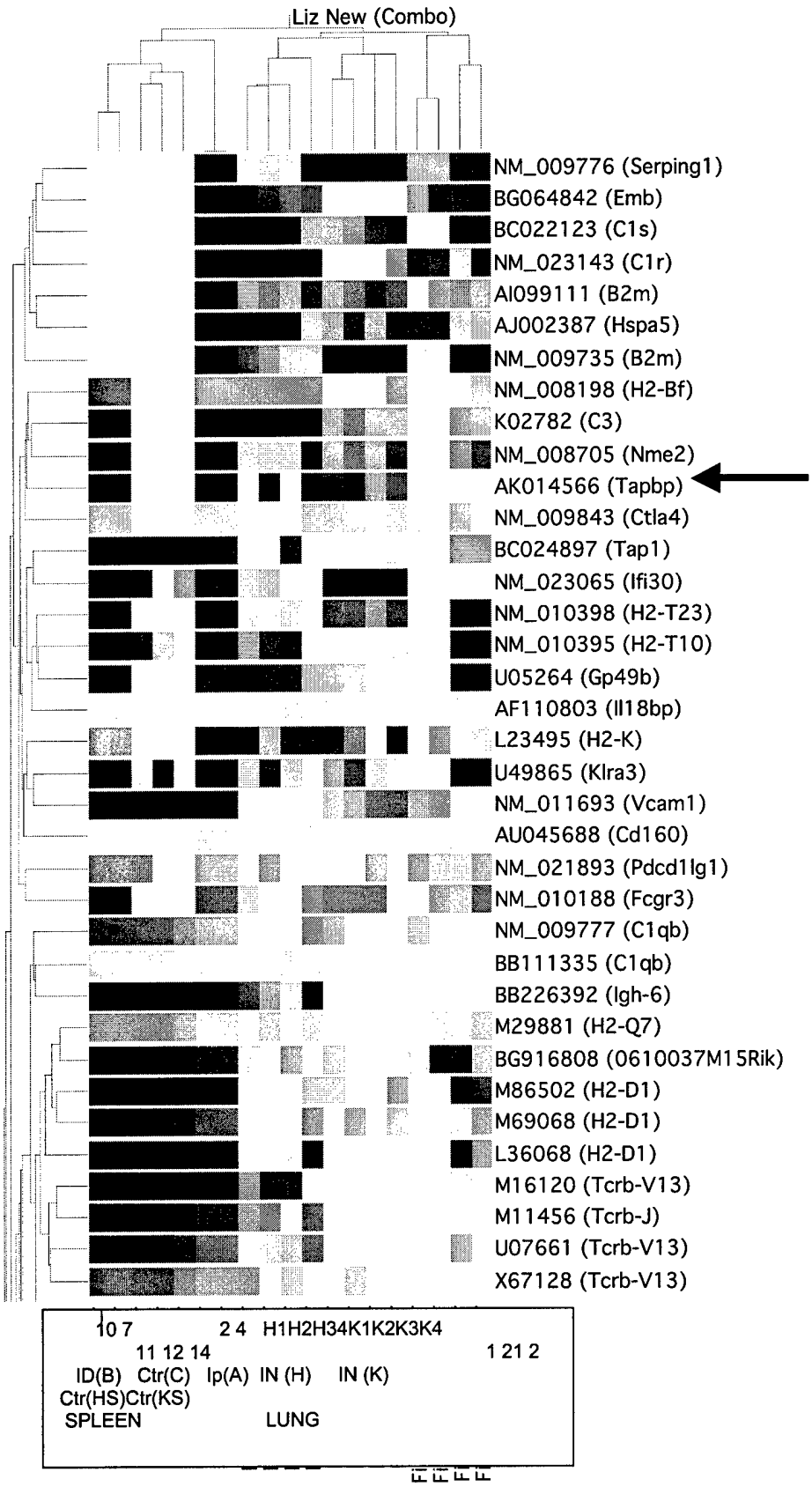
Apoptosis: Apoptosis-related genes were studied using the 430A Chips (arrows indicate genes whose expression is increased in most *F. tularensis* infection conditions in both lung and spleen samples). There are a total of 324 genes in the apoptosis condition. 30 genes were changed by *F. tularensis* infection. The identification of the samples is indicated at the bottom of the expression condition trees.

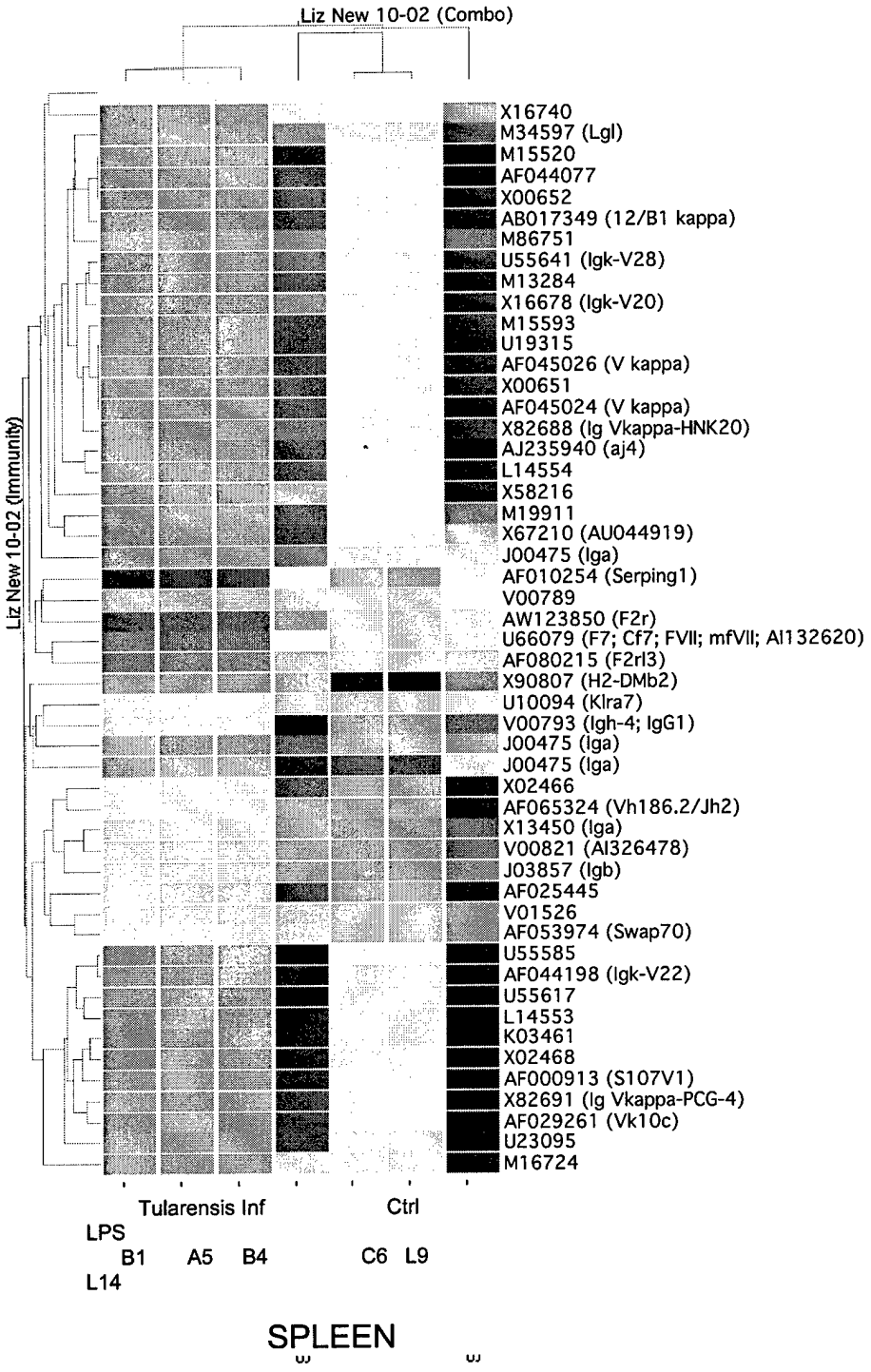


Analysis of Apoptosis Related genes indicates that gene expression clusters and genes of similar treatment cluster together. The F. tularensis spleen RNAs cluster but s branch depending upon route of infection. The intranasal groups clusters very well together with all the C3H mice which are infected intranasally clustering as one group and a second more distinct group is the intranasal infection of the CD14KO strain. Likewise the controls cluster by strain and tissue. The repeat mice in each group cluster within their group indicating that the variation between mice in a group is not significant. Similar studies of apoptosis related genes were performed on an earlier version of an Affymetrix mouse chip, U74A. These are reported below. There were 141 apoptosis related genes and 20 changed following ip or id infection in spleens 4 days post infection.

Immunity: The Condition Tree for genes involved in immunity was examined. In spleen following *F. tularensis* infection many genes involved in antibody production were over-expressed in both experiments with the U74A and 430 chips (10/02 and 8/03). The lungs however did not demonstrate similar gene expression. The lungs were quite distinct from the liver in terms of genes involved in immunity. In general, most of the immunity genes in infected lung were similar to control lung. (Note: all genes with significant change between control and infected were analyzed for all groups. Therefore, while significant fold changes were observed in the spleen for these genes the same was not true for the lungs). On the 430 chips there were 951 genes involved in immunity and

75 were changed (either in lung or spleen) following infection. In the U74A chips there were 172 genes involved in immunity and 52 were changed. The cluster analysis for each chip is shown in the next to condition cluster trees.

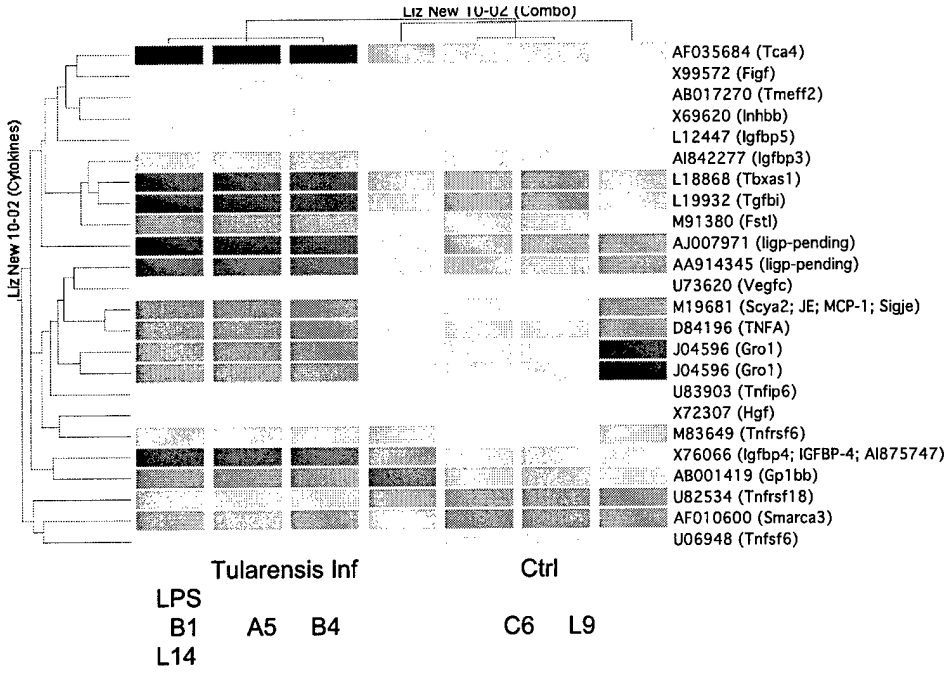

Liz New (Combo)
NM_009776 (Serping1)
BG064842 (Emb)
BC022123 (C1s)
NM_023143 (C1r)
AI099111 (B2m)
AJ002387 (Hspa5)
NM_009735 (B2m)
NM_008198 (H2-Bf)
K02782 (C3)
NM_008705 (Nme2)
AK014566 (Tapbp)
NM_009843 (Ctla4)
BC024897 (Tap1)
NM_023065 (Ifi30)
NM_010398 (H2-T23)
NM_010395 (H2-T10)
U05264 (Gp49b)
AF110803 (Il18bp)
L23495 (H2-K)
U49865 (Klra3)
NM_011693 (Vcam1)
AU045688 (Cd160)
NM_021893 (Pdcd1lg1)
NM_010188 (Fcgr3)
NM_009777 (C1qb)
BB111335 (C1qb)
BB226392 (Igh-6)
M29881 (H2-Q7)
BG916808 (0610037M15Rik)
M86502 (H2-D1)
M69068 (H2-D1)
L36068 (H2-D1)
M16120 (Tcrb-V13)
M11456 (Tcrb-J)
U07661 (Tcrb-V13)
X67128 (Tcrb-V13)
10 7
2 4
H1H2H34K1K2K3K4
11 12 14
1 21 2
ID(B)
Ctr(C)
Ip(A)
IN (H)
IN (K)
Ctr(HS)Ctr(KS)
SPLEEN
LUNG



Selected Gene Tree: Liz New 10-02 (Immu... Colored by: Liz New 10-02
Selected Condition Tree: Liz New 10-02 (Combo) Gene List: [Combo] and [Immunity Protein] (52...

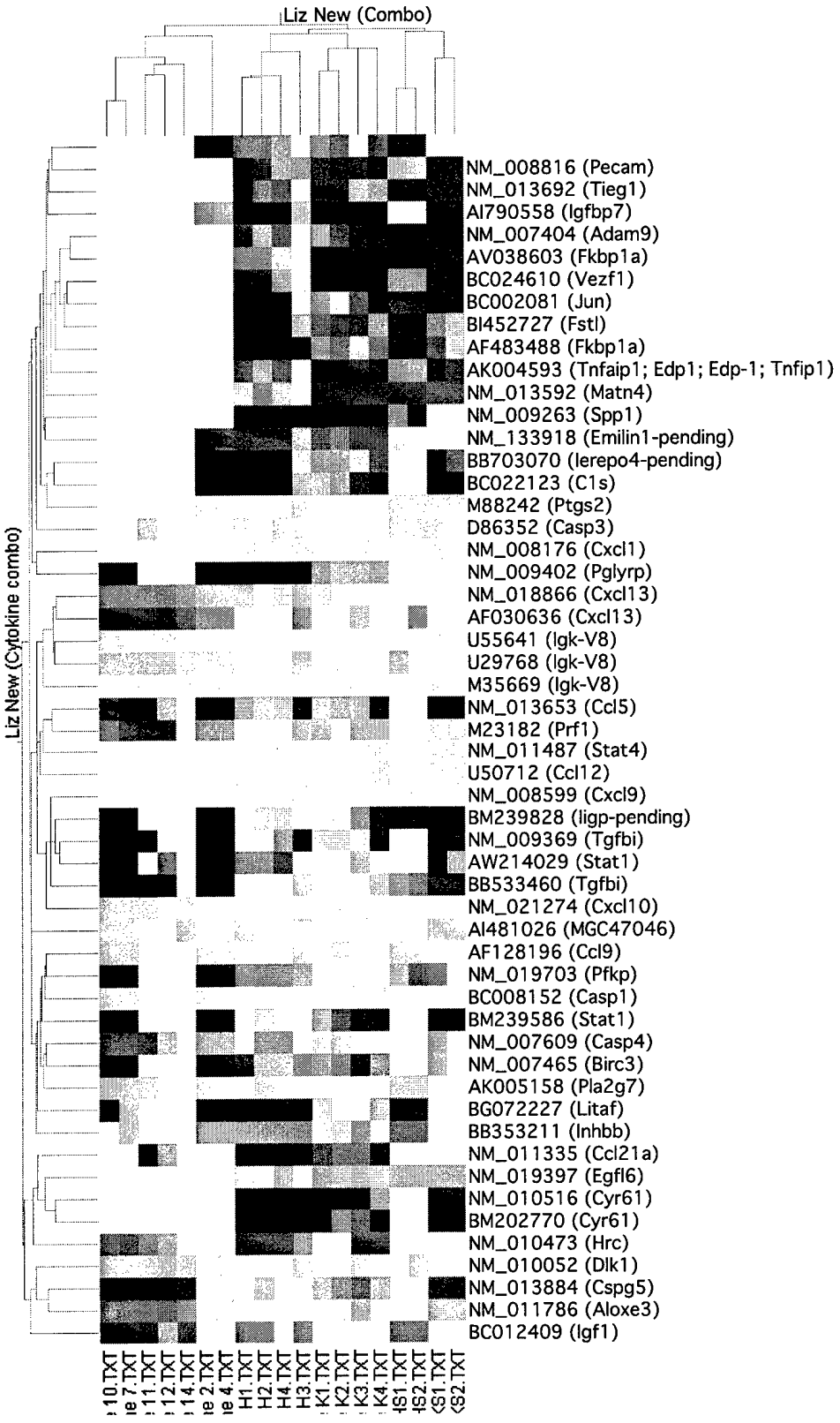
Cytokines

The condition tree was analyzed for cytokine genes. On the 430 chips there were 768 genes in this category and 54 were changed following F. tularensis infection. On the U74A chips there were 151 genes and 24 were changed following infection with F. tularensis. Once again the gene expression of cytokine related genes did not change significantly in the lungs. In contrast, the spleens did change in gene expression much more than the lungs. The gene *Pglyrp* was upregulated in all tissue following tularensis infection. In the category of cytokines the reproducibility between groups of the same treatment was less consistent than for all other gene classes analyzed.



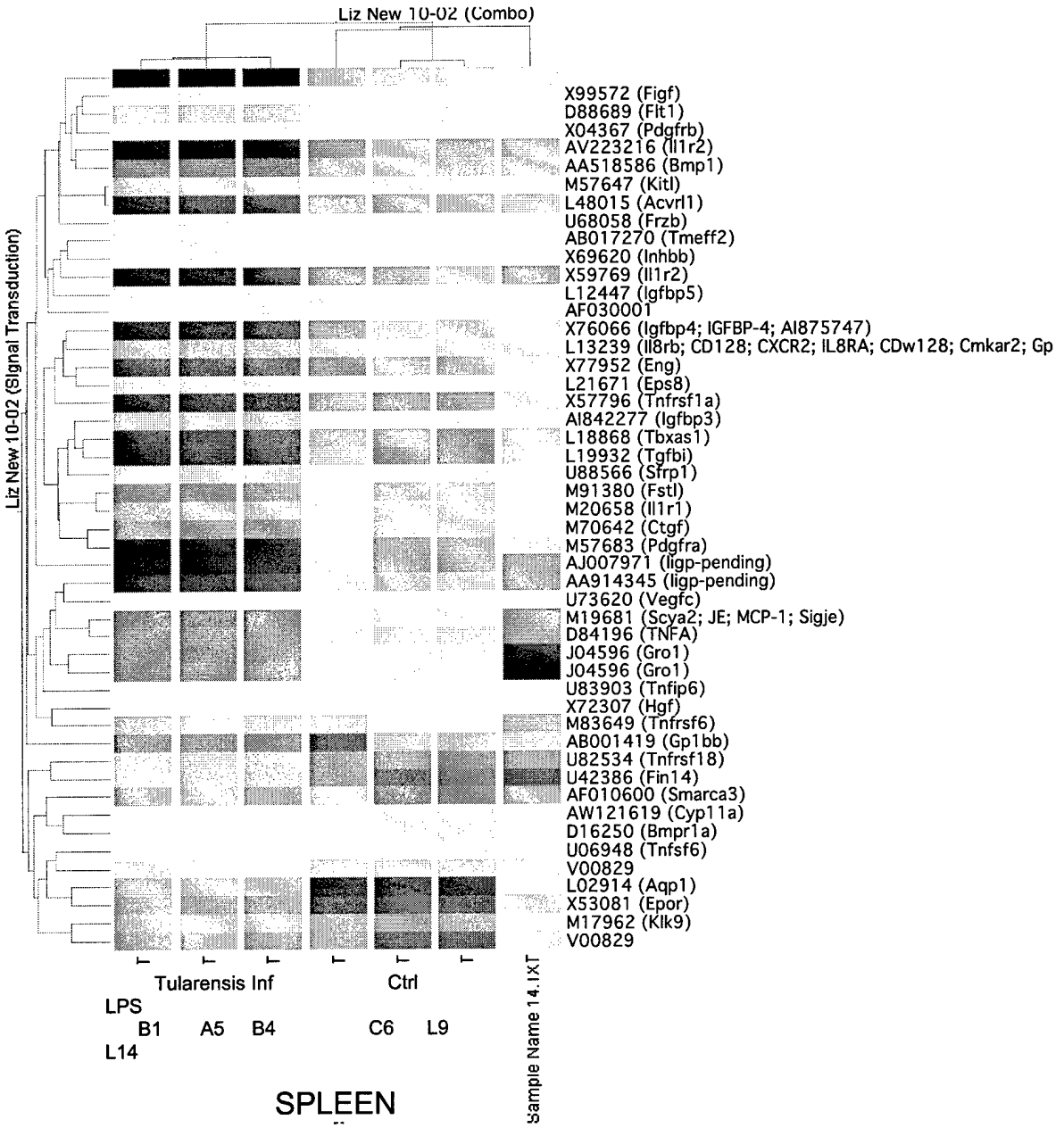
Selected Gene Tree: Liz New 10-02 (Cytokines)
Selected Condition Tree: Liz New 10-02 (Combo)

Colored by: Liz New 10-02
Gene List: [Cytokine] and [Combo] (24), L35049 selected, 1 selected gene not



Selected Gene Tree: Liz New (Cytokine combo) Colored by: Liz New
Selected Condition Tree: Liz New (Combo) Gene List: [Cytokine] and [Combo] (54)

Signal Transduction:
Genes with a 2 fold change were analyzed for the expression of genes related to signal transduction. There are 2145 genes on the 430 chips with 111 changed in response to *F. tularensis*. On the U74 chips there are 453 genes and 49 are changed following *F. tularensis* infection. The reproducibility within treatment groups is very consistent within this gene pool.



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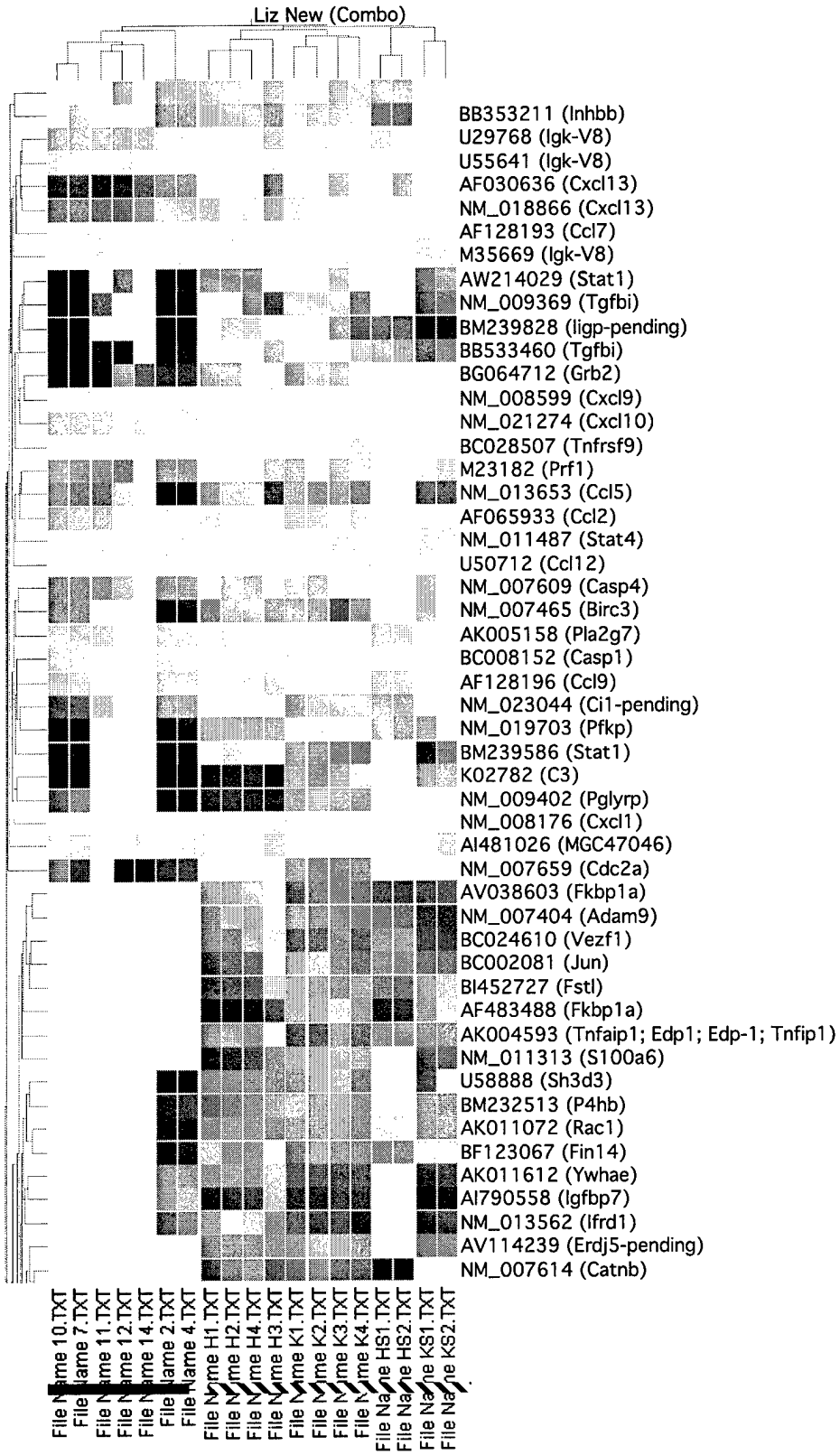
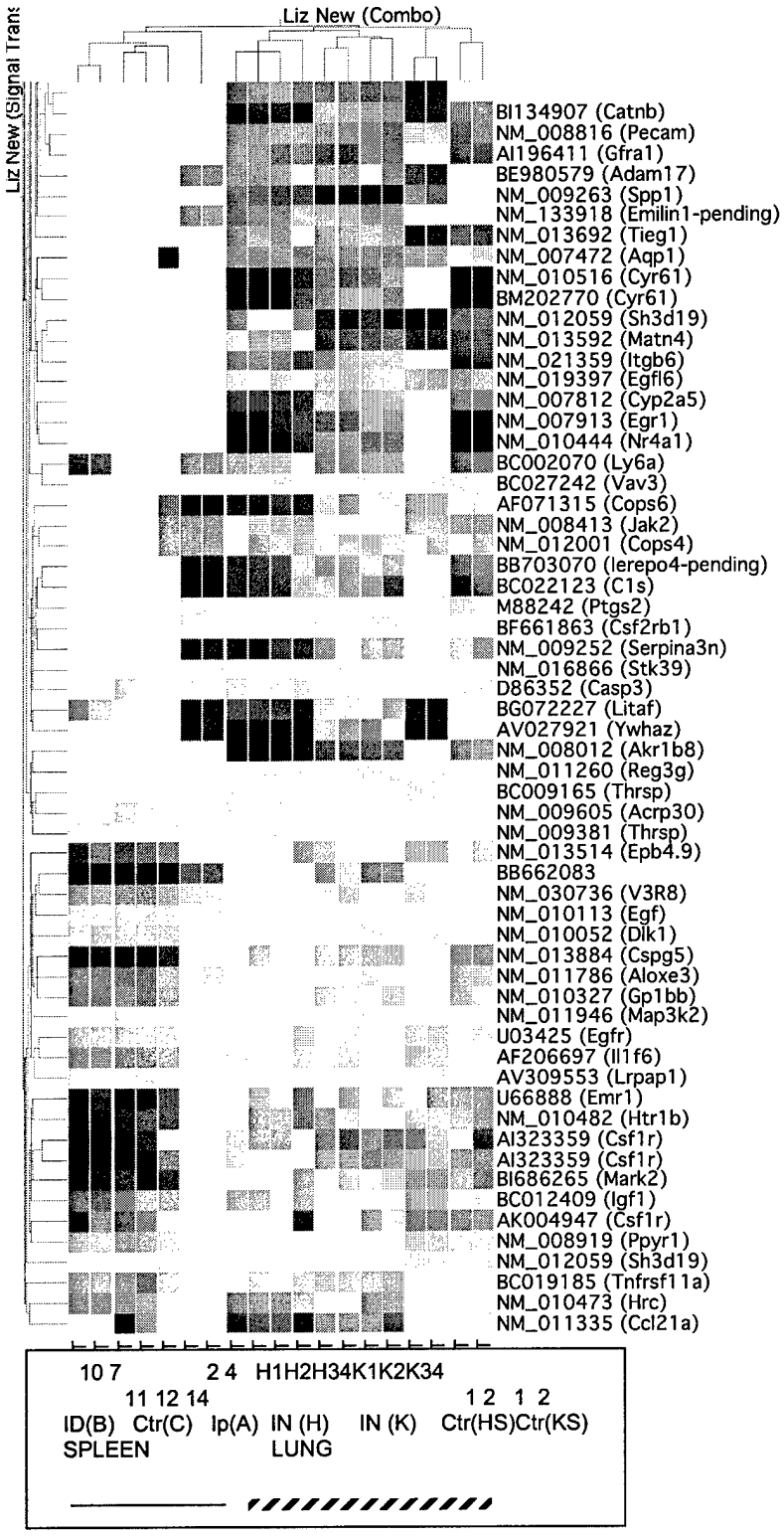
Selected Condition Tree: Liz New 10-02 (Combo) Gene List: [Signal Transducer] and [Combo] (49), L35049 s..



Selected Gene Tree: Liz New (Signal Trans... Colored by: Liz New
Selected Condition Tree: Liz New (Combo) Gene List: [Signal Transducer] and [Combo] (1...



Selected Gene Tree: Liz New (Signal Trans... Colored by: Liz New
Selected Condition Tree: Liz New (Combo) Gene List: [Signal Transducer] and [Combo] (1...

Synopsis of in vivo Mouse/ *F. tularensis* Work:

Top 12 Genes to Study:

Clusterin
Birc3
Granzyme A and B
Pglyrp
G7e
Pancreatic associated genes: Pap and Ppyr
Tapbp
GRO1
CD24
Synuclein
cebpd

The in vivo *F. tularensis*/mouse work has shown that over a thousand genes are altered in response to *F. tularensis* infection with slightly more genes showing increased expression than decreased expression. In addition, the more lethal the infection the greater the number of different genes altered. Transcription profiles of splenic RNA from sublethal infection indicated that most of the genes altered were similar to the subset of altered genes present in the lethal infection with only a few exceptions. The small number of genes that are selectively increased in the sublethal infection may be associated with survival. Target genes for survival which are expressed in the spleen of the sublethal (ip route of infection) are vascular endothelial growth factor C (Vegfc), cytochrome p450 and cebpd. The gene *cebpd* has already been shown to be involved in the response to stress. This gene encodes a transcription factor that regulates cellular growth and differentiation and upregulates proinflammatory genes and genes involved in vascular remodeling. Thus if *cebpd* were selectively upregulated in *F. tularensis* infection, survival may be the outcome. In keeping with this, the upregulation of vascular *Vegfc* may be due to increased levels of *cebpd*, and this pathway has previously been described.

The second finding of note is that the source of the tissue dictates the transcription profiles observed. In the case of spleen samples following *F. tularensis* infection, many of the genes altered deal with red blood cell and hemoglobin metabolic pathways. The spleen transcription profile also involves more genes related to immunity than do samples obtained from lung. Many of the genes induced by *F. tularensis* infection, particularly those associated with B-cell activation and immunoglobulin synthesis, will probably be induced by many infectious agents. Nonetheless, several genes expressed in spleen and lung appear to be unique to *F. tularensis* infection.

The gene *G7e* is upregulated in all *F. tularensis*-infected tissue and linked to the MHC. Granzyme A *(gzma)* and B are upregulated following *F. tularensis* infection in both spleen and lung and these genes encode proteins which mediate the cytotoxic effects of NK, CTL and PMNs and are bactericidal. The expression of these genes may be critical for protection against *F. tularensis* infection. Granzyme A and B are upregulated in both lethal and sublethal infection in the spleen and slightly elevated also in the lungs with and without macrophages (in the intact and the CD14 KO strains). The level of *gzma* is higher in the CD14KO lungs than in lungs from CD14 wild type infected mice suggesting that a compensatory protection system is in place in the absence of macrophages. Another gene involved in innate immunity is *Pglyrp*, and this gene is upregulated in all *F. tularensis* infections and encodes a peptidoglycan recognition protein. The gene, *Gro* encodes a proinflammatory chemokine which is upregulated in *F. tularensis* infection. The gene *C3* (complement protein C3) is elevated in all *F. tularensis* infections.

There are some genes that have similarities among the various *F. tularensis* infection conditions. The top gene of interest is clusterin (*clu*). Clusterin is a gene involved in proliferation and apoptosis regulation, and has anti-apoptotic effects. Clusterin modifies the formation of synuclein, another gene altered by *F. tularensis* infection. Synuclein has been associated with neurotoxicity. Thus, pathways begin to emerge in which several genes on a given pathway are all affected by *F. tularensis* infection. Another gene shared by *F. tularensis* infection is *birc3*. This gene is also anti-apoptotic like clusterin. Interestingly, like clusterin, there is altered expression in lethal and sublethal infection. The gene *birc3* encodes a protein that inhibits apoptosis by binding to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2 and may interfere with activation of ICE-like proteases. This suggests that in lethal infection the cells that are activated by *F. tularensis* infection may not undergo activation-induced cell death and the inflammatory process continues unchecked and repair processes, including erythropoesis and vascular remodeling, do not occur. Anti-apoptotic genes, particularly clusterin and bir3 are target genes to investigate in *F. tularensis* infection.

Section 3. *B. mallei* proteomic analysis
Sub-P.I.s: S. Schutzer; R. Donnelly

As part of our goal to identify signatures and virulence of *B. mallei* by microarray analysis, we wanted to look for complementary and convergent data from a proteomic approach. Towards this aim, we have successfully grown strains of *B. mallei* in chemically defined media (CDM), and demonstrated extracellular proteins with qualitative and quantitative differences. Randomly selected *B. mallei* strains (of 20 stored at –70C) were grown first in standard broth, then subcultured on blood agar plates, and assessed for purity. Subcolonies were inoculated into CDM (composed of amino acids and vitamins, devoid of protein)

that we previously used to grow *Staphylococcus* and *Streptococcus* with concomitant production of virulence factors. We examined the extracellular products of 3 strain colonies, from a phenotypically avirulent isolate and 2 virulent isolates. The CDM media from each culture (after a second passage) at the same OD_{600} was filtered through a 0.22 u filter, assessed for sterility, and then 1 ml was further passed through a 10kd cut-off centrifugal membrane yielding a 10 fold overall concentration of the starting material (previous experiment with unfiltered, unconcentrated solutions did not show proteins possibly due to interfering material such as CDM salts). One ul of each sample was spotted on a hydrophobic Ciphergen protein chip and assessed by SELDI mass spectrometry. In specific regions (10.7kD, 14.3kD) peaks were seen from the sample obtained from the avirulent strain but these peaks were not present when the 2 virulent strains were examined. A 74.5 kD peak was absent in the avirulent strain but was present in both virulent strains. Other peaks of the same mass seemed more prevalent in the avirulent strain sample when compared to a common standard. One could speculate that the absence, or diminished production, of certain proteins in the virulent strains could be relevant to immune evasion. These preliminary experiments demonstrate that our mass spectrometry plan has the potential to detect subtle expression differences between strains.

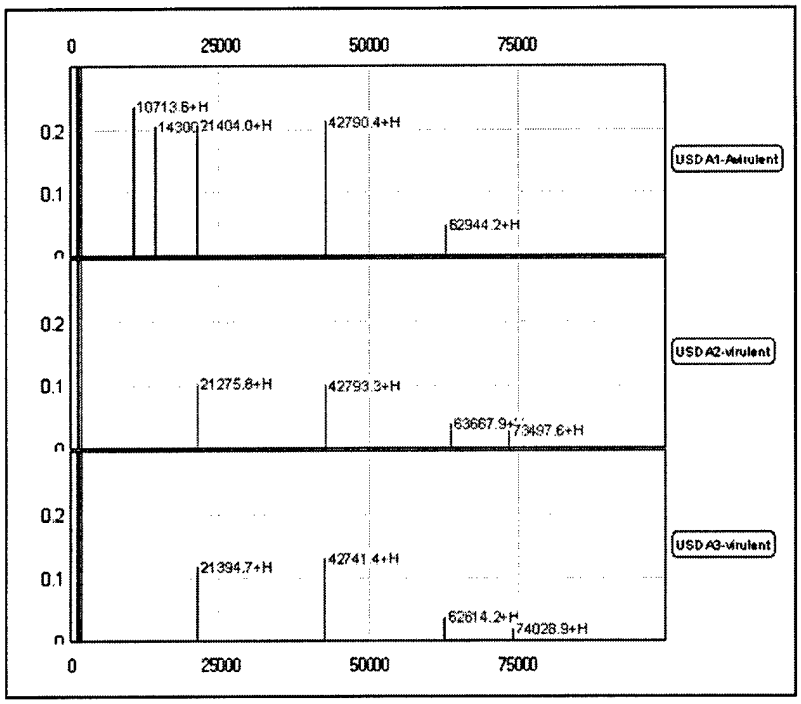


Figure 1. Mass spectrometry detection of extracellular products from virulent and avirulent strains of B. mallei grown in chemically defined media. Peaks of 10.7kD, 14.3kD can be seen from the sample obtained from the avirulent strain but absent in the 2 virulent strains examined. A peak at 74.5 kD was absent in the avirulent strain but present in both virulent strains

Key research accomplishments:

Established whole blood infection model with efficient RNA production protocol.
Identified key genes in human infection with *B. anthracis* virulent and avirulent strains (corroborating published observations).
Identified key, organ-specific genes in *F. tularensis* infections of the mouse that may be unique and diagnostic.
Initiated a proteomics approach to differentiate among strains of *B. mallei* of varying degrees of virulence.

Reportable outcomes: none to date.

Conclusions.

Section 1 (human whole blood infection; microarrays): in the *B. anthracis* infection model, we have identified several genes whose expression is differentially regulated by virulent and avirulent infection. These genes will give us insight into the mechanisms regulating the host's control of avirulent organisms. Furthermore, we hope to identify sets of genes whose expression pattern is unique to each of the bacteria involved, thereby enabling us to distinguish between infections by analysis of gene expression in the blood of infected individuals.

Section 2 (mouse/*F. tularensis*): A panel of key cytokines, signaling pathways and apoptosis-related proteins has been identified in infected mouse tissue. There are some differences in gene expression in different organs as well. The function of these genes in the response the *F. tularemia* infection will be investigated in future work.

Section 3 (*B. mallei* proteomics): we have detected specific differences in the panel of secreted proteins of *B. mallei* strains and efforts are underway to determine the identity of these proteins.

Appendices:

Protocols for whole blood infections and RNA extraction.

Blood Infection Protocol for BW Project

All procedures, unless otherwise noted, are performed in an A2/B3 Biological Safety Cabinent containing an autoclave bag and vesphene disposal container.

- Draw 240ml of blood from healthy volunteer. Blood should be transferred from sterile syringe to a sterile 500ml container aseptically. Take blood to the BL-3.
- Dilute the blood with same volume of RPMI (240ml) utilizing a pipet.
- Aliquot the blood/RPMI mixture into 50ml Oakridge centrifuge tubes with O-rings. Use a pipet to deliver 20ml of the mixture to each nalgene tube.
- Prepare bacteria for infection:
  - Centrifuge cells at 5000RPM for 10 minutes
  - Pull off supernatant with a pipet
  - Re-suspend pellet by pipeting and a quick vortex in RPMI and vortex to mix
- Infect tubes as specified with appropriate bacterium, using a micropipette.
- After infection, all tubes are wiped down with copious amounts of vesphene and moved to the rotisserie in the 37°C incubator. Samples are incubated while rotating slowly for 3 and/or 8 hours.
- Once incubated for the proper time, samples are processed: Whole Blood (WB)
- Please see Ribopure WBC protocol for the rest of the procedure.

Cytoplasmic RNA Preparation w/ NP40 Lysis Buffer

The idea of this method is to isolate the RNA as quickly as possible to avoid any degradation. It is important that the steps up to and including the addition of SDS be done as rapidly as possible. The pellets may then be frozen for further workup.

All micropipetting is performed with aerosol-resistant tips.

All surfaces are decontaminated with vesphene and RNase Away prior to protocol.

1. Resuspend cells in 400 µl Lysis Buffer + 80 units RNAsin (2 µl) using a micropipettor.
2. (Rnasin optional if rapid lysis and preparation times)
3. Transfer cells with a micropipettor to eppendorf tube and vortex gently. Wipe eppendorf tubes with vesphene before micro-centrifuging.
4. Spin eppendorf tubes in microcentrifuge for no more than 30 seconds.
5. Transfer supernatant to new eppendorf tube with micro-pipettor and add + 20 µl 20% SDS (final conc. to ~1%).

6. Add Proteinase K to 250 µg/ml (2µl of a 50 mg/ml solution) with micro-pipettor. Wipe with vesphene before incubating.
7. Incubate 15 - 30 minutes @ 37°C
8. **Phenol:Chloroform extraction 2X**: Transfer solution to a pre-spun phase lock gel tube using a micro-pipettor. Add 400ul of Phenol:Chloroform:Isoamyl 25:24:1 to the phase lock gel tube. Wipe tube with vesphene and transfer to micro-centrifuge and spin at 13,000RPM for 5 minutes. Remove top layer to a new phase lock gel tube and repeat step #8. Transfer final phenol:choloroform:isoamyl extraction to a new phase lock gel tube for chloroform extraction.
9. **Chloroform extraction 1X:** With a micro-pipettor, add 400ul of chloroform to phase lock gel tube. Spin at 13,000RPM for 5 minutes. Transfer top layer of phase lock gel tube to a new 1.5ml eppendorf tube.
10. Add 40 µl 3M NaOAc pH 4.5 – 5.2 with a micro-pipettor.
11. Add 1 mL 100% ethanol with micro-pipettor, wipe with vesphene, and place at -20°C for > 1 hour (can be o/n)
12. Spin at 13,000RPM for 10 minutes @ 4°C or at room temperature in microcentrifuge.
13. Remove ethanol with micro-pipettor and wash with 500ul of 70% ethanol. (critical wash step)
14. Dry pellet by leaving top of eppendorf open and resuspend in 21µl dH_2O when pellet appears to be dry. Resuspend 1ul of sample in 99ul of RNase free water for spectrophotometer. Wipe tubes with vesphene before taking ODs.
15. Wipe eppendorf tubes with vesphene. Store remaining 20ul of sample in –80C.

Lysis Buffer

10 mM Tris pH 7.5
150 mM NaCl
5 mM $MgCl_2$
0.5% NP40 (name change to Ipegal)
add fresh 200 units/ml RNAsin (Promega)

RiboPure WBC Protocol

Pre-Experiment Preparation:

Storage Temperatures for Material Provided in the Kit – only items that are not stored at room temperature are listed here:

- Lysis Solution – 4C
- Acid Phenol Chloroform – 4C
- Sodium Acetate Solution – 4C –20C
- Elution Solution – 4C
- DNase I (RNase Free) -20C
- 20X DNase Buffer –20C
- DNase Inactivation Reagent
- Formaldehyde Load Dye –20C

Items NOT provided with the RiboPure WBC Kit:

- 100% ACS grade ethanol
- 2ml polypropylene tubes that can withstand 13,000rpm
- Whole Blood collection tubes w/ anticoagulant (recommended: potassium EDTA or sodium EDTA)
- Pipettors and RNase free tips
- Heat blocks or incubators at 75C and 37C
- Micro and Clinical centrifuges

Blood Fractionation and WBC Stabilization:

1. Dispense 1.2ml RNAlater into a 2ml tube for each sample.
2. Collect 2.5ml-10ml blood samples
3. Centrifuge samples at 1500-2000 x g for 10-15 minutes at room temperature
4. Remove the plasma with a transfer pipet, being careful not to disturb the WBC's
5. Recover the WBCs in less than 0.5ml by aspiration
6. Put the WBCs into a tube with 1.2ml of RNAlater and mix well.
7. Store the stabilized WBC's or continue with RNA isolation.

WBC Lysis and Initial RNA Purification:

1. Thaw sample and move to 15ml conical (unless already in conical)→ add equal volume PBS to sample, then vortex
2. Centrifuge the WBC and RNAlater mixture for 1min at 13,000 x g or 5000 g for 10 min (with brake)
3. Discard the RNAlater
4. Add 800ul of Lysis Solution and vortex briefly
5. Add 100ul Sodium Acetate Solution and Mix thoroughly by vortexing
6. Withdraw lower phase of 500 ul of Acid Phenol Chloroform, and vortex vigorously for 30 sec.; store the mixture at room temp. for 3 min.; centrifuge for 1 min at max. speed or at 5000 for 10 min (w/o brake) in a microcentrifuge at room temp. to separate the aqueous and organic phases
7. Recover the aqueous phase in a 15 ml conical

Final RNA Purification:

BEFORE: Heat the Elution Sln to 75C in an RNase-free tube; Label the collection tubes; Prepare wash solutions

1. Add 600ul (1/2 volume) of 100% EtOH to each sample, then vortex briefly but thoroughly
2. Pass the sample through the filter cartridge → only 700ul at a time
3. Wash with 700ul of Wash Solution 1
4. Wash with 2 x 700ul of Wash Solution 2/3; after discarding second wash from Collection Tube, replace Filter Cartridge into same Collection Tube and spin assembly for 1 min. to remove all residual fluid from filter
5. Elute RNA with 2 x 75preheated Elution Solution → recover RNA by centrifugation for app. 30 sec at max. speed - (to increase RNA yield slightly (20-30%), wait 1 min. between adding Elution Solution to filter and centrifuging); repeat with second 75 ul of Elution Solution,
   collection RNA in same tube – spin for ~1 min to
   collect all fluid
6. Treat the eluted RNA with DNase I to remove
   contaminating genomic DNA- add 7.5 ul 20X DNase Buffer and 1 ul DNase 1 to the RNA (table)
7. Can bring out of BSL3 at this point
8. Incubate 30 min at 37 °C; Add DNase Inactivation Reagent equal to 20 % of the volume of RNA treated; Store sample at room temp for 2 min; Pellet resin and transfer RNA to a new conical tube – centrifuge for 1 min at max speed to pellet DNase Inactivation Reagent, then transfer the RNA solution to a new RNase-free tube

RNA Clean-Up

Do not use B-ME in Buffer RLT during the clean-up procedure

Centrifuge Phase Lock Gel tubes for 2 minutes at 13,000 RPM

1. Add 200ul 4M Guanidinium Isothiocyantate Solution (4M Guanidinium Isothiocyanate, 25mM Sodium Citrate, p 1M B-ME) to 0.5 x 10^4 – 1 x 10^6 cells
2. Homogenize cells by pipetting up and down several times
3. Transfer all of the homogenate to the pre-spun PLG 2ml heavy tube
4. Add 20ul 20M Sodium Acetate, pH 4.0 to sample. Re-cap PLG tube and mix briefly.
5. Add 200ul of water saturated phenol to the sample. Invert to mix – DO NOT VORTEX
6. Add 60ul chloroform/isoamyl alcohol to sample in the same PLG tube. Invert to mix – DO NOT VORTEX
7. Incubate on ice for 10 minutes
8. Centrifuge at 12,000 – 16,000 x g for 5 minutes
9. Add 200ul phenol/chloroform/isoamyl alcohol to the aqueous phase in the same PLG tube. Invert to mix – DO NOT VORTEX
10. Centrifuge at 12,000 – 16,000 x g for 5 minutes
11. Transfer aqueous to a new phase lock, add equal volumes of chloroform.
12. Centrifuge at 13,000 rpm for 5 minutes.
13. Collect the resulting aqueous phase to Rnase-free microcentrifuge tube. Add an equal volume of 100% isopropanol. Invert to mix
14. Centrifuge at 12,000 – 16,000 x g for 20 minutes
15. Discard the supernatant
16. Wash the pellet several times with 200ul of 70% EtOH. Centrifuge at 12,000 – 16,000 x g for 2-3 minutes to re-pellet the sample
17. Discard the final wash – dry pellet at room temperature
18. Dissolve pellet in Rnase-free water.
19. Store at –70°C